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# Suberized Boundary Zones and the Chronology of Wound Response in Tree Bark

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## ABSTRACT

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Wound response in mechanically injured outer bark of thirteen species of woody dicotyledonous angiosperms was assessed quantitatively by using microscopic photometry/fluorometry and new histochemical techniques. In all species, a morphologically distinct boundary zone formed prior to initiation of new phellogen. The boundary zone tissue, formed from cells present at the time of wounding, was comprised initially of cells with lignified walls. These cells with time developed intracellular suberin linings that imparted an impervious quality to the boundary zone. New phellogen and its derivatives formed immediately internal to this impervious tissue. Based on these findings, a revised wound response chronology for tree bark is proposed.

*Additional key words*: lignin, periderm formation, phellogen generation.

Cell wall structure and composition in tissues formed in tree bark after wounding or infections but prior to wound periderm formation are poorly understood. These tissues have been assigned many names including "non-suberized impervious tissue" (17,18), "wound periderm induction barrier" (9), "ligno-suberized zone" (10), "suberized parenchyma" (1), "lignified zone" (23), and more recently, "impervious tissue" (2–4).

The concepts regarding the chronology of wound healing were initially developed with sweet potato (1), and later studies confirmed and elaborated upon earlier observations (14,16,20). Microscopic examination of wounded potato tissue has shown that the first observable change following mechanical injury was desiccation of several layers of the outermost parenchyma cells exposed to the air (25). Subsequently, a progressive lignification and suberization of parenchyma cells takes place beneath the desiccated cells. Suberization thus provides a barrier to further moisture loss and impedes microbial invasion (13). The final event in the wound-healing process is the generation of a phellogen that gives rise to suberized phellem or cork beneath the suberized parenchyma cells. With potatoes, the wound is considered "healed" when three to seven phellem layers are present (24).

In trees, Mullick described a nonsuberized impervious tissue formed prior to new phellogen in conifers (17,18). At the time, those findings were extremely important because they described a previously unknown tissue and demonstrated a fundamental difference in wound response between woody and herbaceous plants. However, with the use of new, more sensitive and flexible procedures (2,3), I have been unable to confirm Mullick's (18) and Soo's

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(21) conclusions regarding nonsuberized impervious tissue in angiosperms.

The purpose of the present study was to describe the sequence of tissue changes following wounding in tree bark and to present a revised chronology of wound response to facilitate anatomical and disease resistance studies in trees.

## MATERIALS AND METHODS

**Plant material.** Bark samples for histology were taken from *Acer saccharum* Marsh., *Amelanchier arborea* (Michx. f.), *Betula papyrifera* Marsh., *Castanea dentata* (Marsh.) Barkh., *Fraxinus americana* L., *Morus rubra* L., *Ostrya virginiana* (Mill.) K. Koch, *Prunus avium* (L.) L., P. persica (L.) Batsch, *P. serotina* Ehrh., *Quercus alba* L., *Q. rubra* L., and *Tilia americana* L. The trees, located in a mixed hardwood stand near Jordan Station, Ontario, ranged in age from approximately 20 to 60 yr old. *Prunus persica* and *P. avium* trees were orchard grown and were 5 and 15 yr old, respectively. Samples of *C. dentata* were from healthy stump sprouts approximately 10 cm in diameter.

**Wounding and sampling.** All trees were wounded in mid-July, 1983, which was followed by a second wound treatment in mid-August, 1983. A 4-mm-diameter cork borer was used to injure the bark to where green tissue could be observed. An effort was made to injure only the current active phellogen so that vascular cambial responses would not be confounded with phellogen generation. Six wounds were located on the main trunk starting at 1 m above the soil surface and spaced 10 cm apart in a semihelical pattern around the northeast side of the tree. Wounds on *P. avium* were located on a major scaffold limb approximately 15 cm in diameter. Three trees of each species were included in the study. The same three trees were used in both the July and August wound tests, except *P. persica* and *P. avium* for which previously unwounded trees were used for the second replication.

Wounds were sampled with a 2.5-cm-diameter arc punch to remove a bark disk supporting the injured tissue. Samples were taken at 3, 7, 10, 17, and 24 days after wounding. Nonwounded tissue was included at the beginning of the experiment and at each sampling date. The excised bark disks were placed on ice in plastic bags in a Dewar flask, transported to the laboratory, and subjected to Mullick's F-F test (17).

At the conclusion of the F-F test (usually 6 days), the bark disks were halved longitudinally and one half was fixed in FAA (12), prepared as described previously (2), embedded in paraffin, and was sectioned longitudinally on a rotary microtome at 10 $\mu$m for further investigations.

**Histochemistry and photometry.** Assessment of lignin was accomplished by using the phloroglucinol + HCl reaction (11). Tissues were allowed to react for 5 min prior to photometric measurement using percent transmission at 546 nm and the multiple plug or core method described by Mendelsohn (15). Serial sections not treated with phloroglucinol were used as blanks to minimize interference caused by deposition of oxidized tannins or cell wall polysaccharides. Suberin was quantified from tissues treated with phloroglucinol + HCl by measuring autofluorescence intensity at 430 nm (2,3) using epifluorescence fluorometry and the multiple plug method. Percent transmission and autofluorescence intensity were measured with a Leitz MPV compact microscope photometer with stabilized power supplies for the halogen and mercury lamps.

For both lignin and suberin measurements, the measuring diaphragm of the photometer was adjusted to include a 125-$\mu$m-diameter circular area when examined at ×250. This size was chosen because it included a major portion of the boundary zone of most species as it appeared at 24 days post-wounding. All measurements were taken in the phelloderm/cortex region under the original injured periderm. At the early stages of boundary zone formation (3 or 7 days postwounding) when well-defined lignified cell walls were not easily observed, measurements were taken in the phelloderm/cortex region approximately 800 $\mu$m internal to the wound margin. Prior to photometry, all slides were rinsed in 0.5 M NaOH for 30 sec followed by 5 min in running water to remove the complexed F-F reagents. Each photometric value for analysis was the average of five observations taken from five serial sections.

**Statistical analysis.** The data from July and August were combined and each species was analyzed separately by using a one-way analysis of variance to assess differences in lignification and suberization over time. Variation among species was not considered in the experimental design. At each wound age for each species, differences were separated by using Duncan's new multiple range test ($P = 0.05$).

## RESULTS

All species examined formed an impervious boundary approximately 800 $\mu$m internal to the wound surface within the 24-day wound sampling period. Use of the F-F test resulted in all species being rated as forming impervious tissue at either 17 or 24 days postwounding. Histological examination of the tissues, however, revealed that previous interpretations of impervious tissue formation were incomplete. Wounds of all species showed cellular changes indicative of boundary zone formation approximately 800 $\mu$m below the wound surface 3 days after wounding. Initial cellular changes, usually associated with phloem ray parenchyma, included hypertrophy and formation of distinctly visible nuclei. As a distinct boundary zone formed under the wound, cells present at the time of wounding on the distal and proximal edges of the new zone developed characteristics of the initial changes just described. The last cells to show these characteristics were cells of the original injured periderm, the phelloderm and phellogen. By the time the latter cell types were exhibiting dedifferentiation, the cells of the phloem ray parenchyma were already lignified, suberized, and impervious to fluid diffusion. In some species, necrophylactic phellem was several cells thick under the wound while, at the proximal and distal margins, no visible cellular changes were apparent. In all species, the impervious tissue was approximately 280 $\mu$m thick, although thickness in number of cells was extremely variable.

Photometric data on lignification and suberization in boundary zone tissues of wounded bark of thirteen woody angiosperms are presented in Tables 1 and 2, respectively. In all thirteen species, accumulation of phloroglucinol + HCl-positive material in cell walls of the boundary tissue preceded intracellular suberization. All species except *P. persica* and *Q. ruba*, displayed lignification in the absence of suberin when examined 7 days after wounding. After 10 days, eight of the thirteen species possessed measureable suberization of the lignified cells although visual assessments detected suberized cells in all species except *A. arborea*. *A. arborea* did not possess boundary zone suberization in the phelloderm/cortex region until 24 days after wounding. In all species, reestablishment of suberin continuity via the suberized impervious tissue was accomplished by day 24. The majority of the suberized cells of the impervious tissue boundary were present as functioning cortical or phloem parenchyma at the time of wounding. Cell hypertrophy prior to lignification was often observed. Cell division prior to boundary zone formation was observed infrequently. *A. saccharum, B. papyrifera, C. dentata, F. americana, O. virginiana*, and *P. persica* showed complete phellogen continuity and necrophylactic periderm formation around the wound by 24 days.

A diagrammatic interpretation of phellogen generation is presented in Figs. 1–5. Data from Tables 1 and 2 for *F. americana* and *P. avium* best reflect the illustrated tissue changes. At 7 days post-wounding (Fig. 1), a lignified/suberized tissue was present centripetal to the wound surface; proximal and distal boundary tissues were becoming lignified. Lignification had not occurred in inner periderm tissues. At 10 days postwounding (Fig. 2), generation of new phellogen and its derivatives were present centripetal to the wound surface, suberization of lignified cell walls had proceeded in the cortical region, and inner periderm tissues had become lignified. Wounds at 14 days (Fig. 3) displayed a progression of tissue changes associated with phellogen activity beneath the wound and towards the distal and proximal portions. Re-establishment of suberin continuity via the impervious tissue was complete. By day 17 (Fig. 4), phellogen continuity had been

reestablished, and by day 24 (Fig. 5), the centrifugal production of phellogen derivatives had crushed the impervious tissues, except near the distal and proximal portions. Nonsuberized lignified cells were always found externally abutting the suberized impervious tissue.

In most species, lignification reached its peak by day 17. Ten of the 13 species showed no significant increase in percent transmission at 546 nm from day 17 to day 24. All species, except *P. serotina* and *Q. rubra*, displayed a consistent increase in suberin autofluorescence intensity after its initial detection.

## DISCUSSION

Reports of lignification as a response to wounds or infection in tree bark are numerous (5–8,21,22). Mullick (17) extensively described a nonsuberized impervious tissue in conifer bark that was located in the same position as the boundary zones described herein. Soo (21), a student of Mullick, reported the occurrence of nonsuberized impervious tissue in woody dicotyledonous angiosperms and showed that cell walls of this tissue were phloroglucinol + HCl-positive. Results of recent studies reported from this laboratory, however, show that imperviousness in woody dicots is more closely related to the formation of intracellular suberin linings (3), and the present study is the first to describe the sequence and location of suberin deposition that occurs during formation of the boundary zone, prior to generation of new phellogen. It is likely that lignin and suberin deposition forms a barrier to apoplastic and symplastic fluid movement, thus preventing tissue desiccation and, perhaps, limiting internal diffusion of enzymes or toxins involved in pathogenesis. In the present study, microorganisms (particularly yeasts) were frequently observed growing on the surface of exposed wounds. Colonization of wounded, dying tissues, as determined by visual examination for fungal hyphae, was observed once (in *A. arborea*) and colonization of the boundary zone tissue was never observed.

Lignified cells in the boundary zone may be visible as early as 3 days after wounding, as was observed with *A. saccharum*. The phloroglucinol-positive substances in *A. saccharum* bark 3 days after wounding were soluble in dilute NaOH, indicating that the reaction was probably due to wall-bound phenolic acids (19). Boundary zone tissue, 7 days of age and and older, from *A. saccharum* wounds did not display solubility in dilute NaOH (*unpublished*). Wall-bound phenolic acids may with time polymerize and form lignin in boundary zone cell walls.

The importance of phenolic compound metabolism and the shikimic acid pathway in suberin biosynthesis has been emphasized

TABLE 1. Light transmission at 546 nm from wound boundary zone tissues in tree bark sampled at varying times after wounding and treated with phloroglucinol + HCl[y]

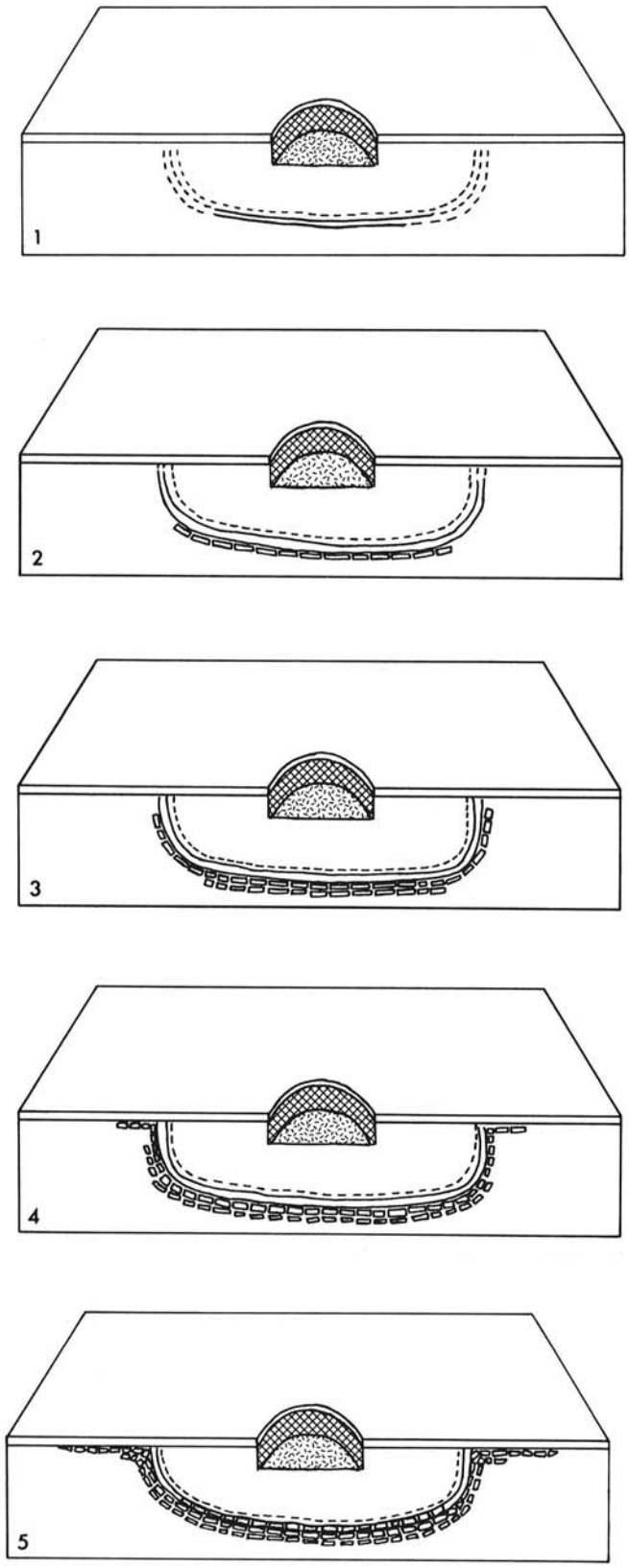
| Tree species | Light transmission (%) at wound age (days): 0 | 3 | 7 | 10 | 17 | 24 |
|---|---|---|---|---|---|---|
| *Acer saccharum* | 99.8[z] a | 83.0 b | 72.5 b | 57.7 c | 56.0 c | 42.2 d |
| *Amelanchier arborea* | 99.9 a | 99.4 a | 94.9 b | 86.8 c | 69.5 d | 69.2 d |
| *Betula papyrifera* | 100.3 a | 93.9 ab | 88.8 bc | 83.1 c | 56.1 d | 62.2 d |
| *Castanea dentata* | 100.1 a | 99.6 a | 85.1 b | 69.7 c | 54.4 d | 51.9 d |
| *Fraxinus americana* | 100.0 a | 97.4 a | 79.9 b | 71.8 c | 55.4 d | 58.2 d |
| *Morus rubra* | 99.7 a | 98.4 a | 95.4 b | 92.0 c | 83.2 d | 72.8 e |
| *Ostrya virginiana* | 100.2 a | 99.0 a | 93.1 b | 78.8 c | 59.1 d | 73.5 c |
| *Quercus alba* | 99.7 a | 97.9 a | 93.2 b | 82.1 c | 75.0 cd | 66.9 de |
| *Q. rubra* | 99.9 a | 99.2 a | 85.3 b | 64.2 cd | 70.7 c | 60.2 cd |
| *Prunus avium* | 100.2 a | 98.7 a | 91.9 b | 87.4 c | 82.1 cd | 80.9 d |
| *P. persica* | 100.1 a | 99.4 a | 96.2 b | 90.1 c | 85.3 cd | 82.0 d |
| *P. serotina* | 100.0 a | 97.5 a | 85.6 b | 72.2 c | 52.4 e | 58.5 d |
| *Tilia americana* | 100.0 a | 97.5 a | 83.9 b | 73.6 c | 68.3 c | 64.5 c |

[y] Percent transmission measured over a circular area 125-$\mu$m in diameter. Leitz interference line filter 546 has a half value of 12 nm.
[z] Different letters in rows denote significant differences according to Duncan's new multiple range test ($P = 0.05$).

TABLE 2. Suberin autofluorescence intensity at 430 nm from wound boundary zone tissues in wood sampled at varying times after wounding, treated with phloroglucinol + HCl, and examined with epifluorescence illumination[w,x]

| Tree species | Autofluorescence at wound age (days): 0 | 3 | 7 | 10 | 17 | 24 |
|---|---|---|---|---|---|---|
| *Acer saccharum* | 0[y,z] a | 0 a | 0.9 a | 1.8 a | 10.8 b | 15.9 b |
| *Amelanchier arborea* | 0 a | 0 a | 0 a | 0 a | 0 a | 0.2 b |
| *Betula papyrifera* | 0 a | 0 a | 0 a | 2.8 a | 11.2 b | 43.3 c |
| *Castanea dentata* | 0 a | 0 a | 0 a | 1.7 a | 6.9 b | 23.7 c |
| *Fraxinus americana* | 0 a | 0 a | 0.8 a | 6.1 b | 13.1 c | 19.1 d |
| *Morus rubra* | 0 a | 0 a | 0 a | 1.8 b | 3.8 c | 7. 8 d |
| *Ostrya virginiana* | 0 a | 0 a | 0 a | 0.4 a | 13.6 b | 41.8 c |
| *Quercus alba* | 0 a | 0 a | 0 a | 0.9 a | 3.0 b | 3.4 b |
| *Q. rubra* | 0 a | 0 a | 1.3 b | 7.9 c | 2.6 b | 11.4 d |
| *Prunus avium* | 0 a | 0 a | 0.3 a | 1.7 b | 7.9 c | 13.0 d |
| *P. persica* | 0 a | 0 a | 7.1 b | 14.4 c | 20.8 d | 22.1 d |
| *P. serotina* | 0 a | 0 a | 1.0 a | 6.9 b | 17.9 c | 9.6 b |
| *Tilia americana* | 0 a | 0 a | 0 a | 3.3 b | 17.4 c | 19.3 c |

[w] Autofluorescence intensity measured over a circular area 125 $\mu$m in diameter.
[x] Fluorescence filter combination 340–380 nm excitation, 430 nm suppression.
[y] Different letters following values within rows denote significant differences according to Duncan's new multiple range test ($P = 0.05$).
[z] 0 indicates no suberin was observed.




**Figs. 1–5.** Diagrammatic representation of the wound response boundary zone and generation of necrophylactic periderm in tree bark. Post-wounding times for the changes illustrated in the figures are approximately 7, 10, 14, 17, and 24 days, respectively, for wounds made in July and August on trees growing in southern Ontario. Dotted lines represent lignified tissues of the boundary zone, suberization of lignified cells is represented by solid lines, and necrophylactic periderm is represented by the rectangular cells immediately internal to the boundary zone. The boundary zone is derived from hypertrophic dedifferentiation of cells present at the time of wounding. The necrophylactic periderm cells are newly formed as part of the wound response.

by Kolattukudy (13). Accumulation of phenolic compounds appears to be a necessary step in the biosynthesis of suberin and the histochemical chronology reported here supports this finding. The aliphatic components of suberin may be esterified to the wall-bound phenolic constituent and, in addition, deposition of waxes may also occur in association with suberin (13). Suberin was never detected histochemically in the first few cell layers of the wound surface as described by Kolattukudy (13). In trees, cell wall changes at the wound surface were usually associated with polysaccharide accumulation which included $\beta$-1,3 linked compounds (callose) (4).

Tissues in various stages of wound response could be observed by sampling at any one time, i.e. at 7, 10, 17, or 24 days after wounding, depending upon species and inherent regenerative capacity. In this regard, the F-F test was not a test for a morphologically discrete impervious tissue, rather it was a test for reestablishment of suberin continuity. Thus, the F-F test detected complete imperviousness, not impervious tissue. Impervious cells did not form synchronously to comprise a tissue which formed a distinct morphological boundary around an entire wounded area. The cells and subsequent tissues formed first underneath wounds, between the wound surface and the vascular cambium, and last in the region of the original phellogen. As the impervious tissue formed, new phellogen completed differentiation and necrophylactic phellem was produced. Often, the tissues beneath a wound possessed a necrophylactic periderm while, at the same time, tissues immediately proximal and distal to the wound (in longitudinal section) showed either no reaction, only lignified cells, a mixture of lignified and ligno-suberized cells, a complete ligno-suberized tissue layer contiguous with the original phellogen, or a completely generated contiguous necrophylactic periderm. It appears that dedifferentiation of extant phelloderm and phellogen may be the limiting factor for rapid generation of new phellogen in some species.

In tissues in which suberin continuity had not been reestablished, the F-F test gave negative results, although a portion of the impervious tissue and new phellogen were well formed. This situation was particularly noticeable in earlier studies of diseased bark (4). Combined use of histochemistry and epifluorescence microscopy was the most reliable method for detecting incomplete or defective impervious boundaries.

Based on this study, a modified chronology of histochemical events in tree bark following wounding is proposed. After wounding, the outermost cell layers become desiccated and die. Suberization at the wound surface is never observed. Approximately 1 mm inward from the wound surface, a lignified boundary zone of variable thickness forms between the wound surface and the vascular cambium. Before the new phellogen is completely differentiated, the innermost cells of the lignified zone develop an intracellular suberin lining to comprise a tissue approximately 280 $\mu$m thick. The new phellogen then completes differentiation immediately inside the impervious boundary. Cells in the external portion of the lignified zone do not develop suberin linings and, therefore, are not completely impervious to fluid diffusion. With time, formation of the boundary proceeds to eventually reestablish continuity of suberized tissues, first via the ligno-suberized boundary tissue, and, second, via formation of a new phellogen and its suberized derivatives.

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